Genetics and Molecular Research

Molecular identification and genetic variation of varieties of *Styphnolobium japonicum* (Fabaceae) using SRAP markers

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Genet. Mol. Res. 15 (2): gmr.15027837
Received October 15, 2015
Accepted January 18, 2016
Published May 6, 2016
DOI http://dx.doi.org/10.4238/gmr.15027837

ABSTRACT. Thirty-four *Styphnolobium japonicum* varieties were analyzed using sequence-related amplified polymorphism (SRAP) markers, to investigate genetic variation and test the effectiveness of SRAP markers in DNA fingerprint establishment. Twelve primer pairs were selected from 120 primer combinations for their reproducibility and high polymorphism. We found a total of 430 amplified fragments, of which 415 fragments were considered polymorphic with an average of 34.58 polymorphic fragments for each primer combination. The percentage of polymorphic fragments was 96.60%, and four primer pairs showed 100% polymorphism. Moreover, simple matched coefficients ranged between 0.68 and 0.89, with an average of 0.785, indicating that the genetic variation among varieties was relatively low. This could be because of the narrow genetic basis of the selected breeding material. Based on the similarity coefficient value of 0.76, the varieties were divided into four major groups. In addition, abundant and clear SRAP fingerprints were obtained and could be used to

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establish DNA fingerprints. In the DNA fingerprints, each variety had its unique pattern that could be easily distinguished from others. The results demonstrated that 34 varieties of *S. japonicum* had a relatively narrow genetic variation. Hence, a broadening of the genetic basis of breeding material is necessary. We conclude that establishment of DNA fingerprint is feasible by means of SRAP markers.

Key words: *Styphnolobium japonicum*; SRAP; Molecular identification; Genetic variation; DNA fingerprints

INTRODUCTION

Styphnolobium japonicum L. Schott (syn. *Sophora japonica* L.) is a tree species belonging to the family Fabaceae. It is native to China and Korea, and is a source of timber, medicine, nectar, and ornamental material. It was formerly included within a broader interpretation of the genus *Sophora* (Käss and Wink, 1996; Santamour Jr and Riedel, 1997). Because it lacks the ability to form symbioses with rhizobia (nitrogen fixing bacteria) through the roots, it has been redefined as the genus *Styphnolobium*. It is also known as Chinese scholar tree or pagoda tree and has been cultivated for more than 3000 years in China. Owing to its potential values in pest and disease resistance, pollution tolerance, microclimate regulation, and high adaptability (Tang and Jiang, 2006), it is extensively used as a street tree. It has therefore been regarded as a city tree of Beijing, Xi'an, Dalian, Taian, and other cities in China.

During the last few decades, many new varieties of *S. japonicum* have been developed. This imposes new challenges for variety identification and protection of plant breeder's rights (PBR). The available morphological characteristics are limited in quantity and easily influenced by environmental factors, which means that they are time-consuming to measure, and therefore, have a limited application in variety identification (Tseng, 1962; Smith and Smith, 1989; Ma and Cai, 1996). In contrast, molecular marker technology based on genome DNA fingerprinting can overcome the disadvantages mentioned above (van de Wiel et al., 1999).

With rapid development of modern biotechnology, many molecular markers have been developed. Among them, sequence-related amplified polymorphism (SRAP) markers have been developed in *Brassica* species (Li and Quiros, 2001). These have several advantages over other markers, such as ease of use, reasonable throughput rate, high polymorphism, low cost, disclosure of numerous co-dominance loci, targeting open reading frames (ORFs), and easy isolation of bands for sequencing (Ren et al., 2004; Ai et al., 2011). Previous studies have shown that SRAP markers are evenly distributed in the genome, and could generate higher polymorphism than markers such as ISSR, RAPD, and SSR (Budak et al., 2004). SRAP markers have been used in genetic map construction (Gao et al., 2008), variety identification (Liu et al., 2008; Wu et al., 2010), and constructing DNA fingerprints in varieties, such as herbaceous peony (Guo et al., 2011), *Porphyra* lines (Qiao et al., 2007), turf-type *Cynodon* (Wang et al., 2009), and *Hemarthria compressa* "Guangyi" (Huang et al., 2014).

In this study, DNA fingerprints and dendrograms of *S. japonicum* were established using SRAP markers. The aim was to investigate molecular identification and genetic variation among varieties of *S. japonicum*, which may provide a scientific basis for breeding and utilization of these varieties. The goal was to apply DNA fingerprints using SRAP markers as a useful tool to provide evidence for protection of PBR in infringement cases.

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MATERIAL AND METHODS

Plant materials

Thirty-four varieties, including one sample tree of local *S. japonicum* in Fengtai of Beijing as a control material, were used in this study (Table 1). Trees of the different varieties were planted in a nursery located in the Fengtai District of Beijing. Three young compound leaves were collected from three sample trees for each variety and stored for DNA extraction.

Table 1. List of the names of all varieties used.

Abbreviation	*S. japonicum* variety name	Abbreviation	*S. japonicum* variety name
CK	Local tree in Fengtai	CL7	'Clone 21'
WS	'Wuse'	CL8	'Clone 24'
JZ	'Golden stem'	CL9	'Clone 30'
JY	'Golden leaf'	CL10	'Clone 33'
LZ	'Pendula'	CL11	'Clone 34'
LD	'Liaohong'	CL12	'Clone 35'
C1	'Caozhou 1'	CL13	'Clone 40'
C2	'Caozhou 2'	CL14	'Clone 48'
C3	'Caozhou 3'	CL15	'Clone 53'
SJ	'Shuangjimi'	CL16	'Sun 19'
HD	'Oligophylla'	CL17	'Sun 20'
CL1	'Clone 2'	CL18	'Sun 27'
CL2	'Clone 4'	CL19	'Wang 21'
CL3	'Clone 12'	CL20	'Huang a'
CL4	'Clone 13'	CL21	'Huang b'
CL5	'Clone 18'	CL22	'Qingchao 1'
CL6	'Clone 20'	CL23	'Guo 1'

DNA extraction

Total genomic DNA was extracted using the CTAB procedure (Murray and Thompson, 1980). The quality of the DNA samples was measured using an electrophoresis and imaging analysis system and the concentration was determined using a spectrophotometer Nanodrop 8000 (Thermo Scientific, Wilmington, NC, USA). Finally, the extracted DNA was diluted to a working concentration of 50 ng/μL and stored at -20°C in a freezer.

SRAP-PCR amplification

In this study, a total of 120 SRAP primer combinations containing 10 forward primers and 12 reverse primers (Table 2) (Li and Quiros, 2001; Ahmad et al., 2004) were initially screened for polymorphism, using samples from four varieties. All primers were synthesized in Sangon Biotech Co. Ltd. (Shanghai, China). Preliminary optimization of SRAP-polymerase chain reactions (PCR) was performed as follows: 2 μL 10X Taq buffer (Mg^{2+}-free), 0.30 mM dNTP, 1.0 U Tap polymerase, 90 ng DNA, 0.2 μM of each primer, 2.0 mM Mg^{2+}, and sterile double-distilled water to reach the total volume 20 μL. DNA amplifications were performed with an initial step at 94°C for 5 min, which was followed by five cycles of 1 min at 94°C, 1 min at 35°C, and 1 min at 72°C. The following 35 cycles included 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min, followed by final storage at 4°C (Li and Quiros, 2001). All the SRAP-PCRs were performed in the same thermal cycler (Bio-Rad, Miami, Florida, USA). The final PCR products were detected using a capillary electrophoresis analyzer (Qiagen, Dusseldorf, Germany).

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Table 2. Primer sequences used for the SRAP analysis.

Primer code	Forward primers (5'-3')	Primer code	Reverse primers (5'-3')
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCTTTCCGGTAA	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTTG	Em7	GACTGCGTACGAATTCAG
Me8	TGAGTCCAAACCGGTCA	Em8	GACTGCGTACGAATTCCA
Me9	CGAATCTTAGCCGGATA	Em9	GACTGCGTACGAATTGCC
Me10	CGAATCTTAGCCGGAGC	Em10	GACTGCGTACGAATTTCA
		Em11	GACTGCGTACGAATTGAG
		Em12	GACTGCGTACGAATTCAT

Data analysis

SRAP is a co-dominant marker. The capillary electrophoresis products were scored as "1" and "0", where "1" indicated the presence of a specific allele and "0" indicated its absence, to generate a binary matrix. The similarity matrix and dendrogram were constructed using the numerical taxonomy multivariate analysis system (NTSYS-pc) v. 2.1 (Exeter Software, Setauket, NY) software package (Rohlf, 2000). A cluster analysis was first conducted, based on the similarity coefficients, using the unweighted pair group with arithmetic average (UPGMA) method with the SAHN module. The reliability of the dendrogram was then assessed by estimating the cophenetic correlation and comparing it with the similarity matrix, using the MxComp module of NTSYS-pc v. 2.1 (Rohlf, 2000). The result of this test was used to investigate how well the dendrogram represented the similarity data.

RESULTS

Selection and analysis of SRAP primer combinations

Twelve primer pairs were selected from 120 SRAP primer combinations, based on their reproducibility and polymorphism, to identify the 34 varieties of *S. japonicum*. The amplification fragment size mainly ranged between 100 and 2000 bp. A total of 430 amplified fragments were detected, out of which 415 fragments were considered polymorphic. For each primer combination, the number of polymorphic fragments averaged 34.58, and ranged from 23 to 51. The average percentage of polymorphic fragments was 96.60% and four primer pairs showed a polymorphism of 100% (Table 3).

Construction of SRAP-DNA fingerprints for varieties

The primer combination Em6-Me7 was used to construct SRAP-DNA fingerprints for the 34 varieties (Figure 1). During the construction of the DNA fingerprints, fragments less than 100 bp were rejected, because they might be primer dimer. Polymorphic fragments were mainly distributed above 300 bp. Using the SRAP-DNA fingerprints, each of the varieties displayed its own unique fingerprinting pattern and could easily be distinguished from the others. By using the fingerprints, it was easy to identify whether a test sample was identical or similar to any of the 34 varieties.

Table 3. Polymorphism amplified by SRAP primer combinations in 34 varieties.

Code	Primer combination	No. of total fragments	No. of polymorphic fragments	Percentage of polymorphic fragments (%)
1	Me1–Em4	51	50	98.04
2	Me1–Em5	40	38	95.00
3	Me1–Em7	31	29	93.55
4	Me3–Em7	28	28	100.00
5	Me3–Em12	32	32	100.00
6	Me6–Em2	27	26	96.30
7	Me6–Em7	40	40	100.00
8	Me6–Em10	23	22	95.65
9	Me9–Em3	32	32	100.00
10	Me9–Em12	34	32	94.12
11	Me10–Em8	44	39	88.64
12	Me10–Em9	48	47	97.92
Total		430	415	
Average		35.83	34.58	96.60

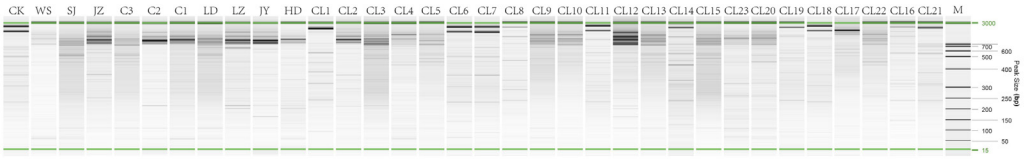

Figure 1. SRAP fingerprints of the Em6-Me7 primer combination for all varieties. Letters above the figure are the abbreviations of 34 varieties; numbers on the right side of the figure are the sizes (bp) of markers.

Genetic variation and cluster analysis

The differences among the 34 varieties of *S. japonicum* at the DNA level were determined by comparing their genetic similarity coefficients, according to the detected SRAP data. The genetic similarity coefficients were computed based on the proportion of shared fragments, and ranged from 0.68 to 0.89, with an average of 0.785. This indicated a narrow genetic basis and a high similarity among the varieties. A high similarity coefficient indicates a closer genetic relationship, and vice versa. The highest genetic similarity coefficient (0.89) was found between CL15 and CL16, and the lowest value was detected between CL9 and LZ.

A dendrogram (Figure 2) was constructed based on the similarity matrix (data not shown).

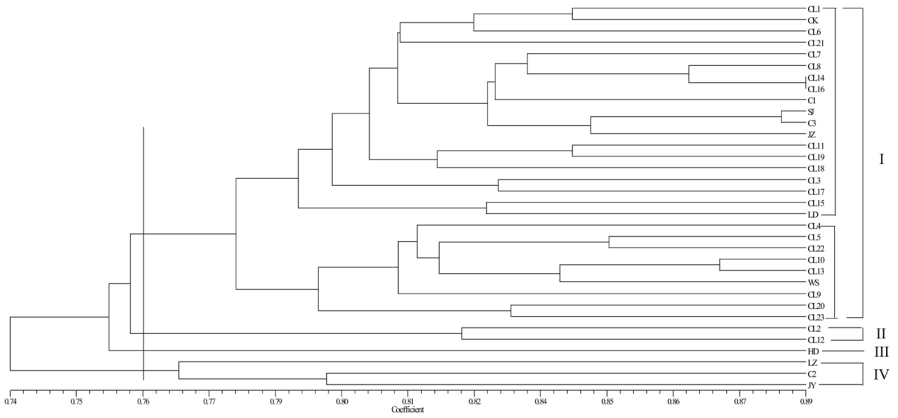

Figure 2. Dendrogram generated using the UPGMA method based on SRAP markers.

The cophenetic correlation between the ultrametric similarities of the cluster tree and the similarity matrix was estimated at 0.80, suggesting that the dendrogram well represents the similarity matrix. With a genetic similarity coefficient value of 0.76, the 34 varieties were divided into four major groups. Group II included two varieties: CL2 and CL12; Group III included HD; Group IV consisted of three varieties: LZ, C2, and JY; the all other varieties were classified into Group I. Group I was further separated into two subgroups.

DISCUSSION

This is the first study focused on the molecular identification and genetic variation among 34 varieties of *S. japonicum* using SRAP markers. The similarity coefficients, ranging from 0.68 to 0.89, demonstrated that the 34 varieties had a relatively low genetic variation. Therefore, it is necessary to broaden the genetic basis of breeding materials. The low genetic variation detected may be attributable to breeding and selection within a limited genetic basis over the long period of cultivation. However, it was evident that the percentage of polymorphic fragments was high (96.60%), higher than in e.g. *Robinia pseudoacacia* L. (93.41%) (Sun et al., 2009). The high polymorphism may be partially attributed to the large genetic differences among some of the varieties, as well as the advantages of the SRAP markers, such as high reproducibility, polymorphism, and suitability to amplify ORFs (Ai et al., 2011).

Compared to other varieties, the HD variety, also known as 'Wuyehuai', was separated into a single cluster, owing to large differences in leaf morphology compared to other varieties. The three varieties C1, C2, and C3 were clones developed from 153 plus trees selected throughout the Shandong province. They share many morphological characteristics but the cluster analysis revealed that C1 and C3 were closer to each other and both differed greatly from C2. This could likely be explained by differences in their geographic origins. Although it is difficult to distinguish C1, C2, and C3 based on morphological characteristics, they could be distinguished at the DNA level using the SRAP-DNA fingerprints based on the Em6-Me7 primer combination. A striking finding was that the LZ and JY varieties that are very distinct morphologically, clustered in a same group, indicating that the genetic distance between them was rather small. Further studies are needed to fully understand the underlying causes.

Compared with polyacrylamide gel electrophoresis, capillary electrophoresis is more effective and stable in detecting PCR products, which reduces human and system errors. Our findings indicate that SRAP markers are suitable for the analysis of genetic variation and molecular identification among varieties of *S. japonicum*, even for materials with a narrow genetic basis.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Special Fund for Public Interest (#201204307) from the State Forestry Administration. We thank Yan He at Beijing Municipal Bureau of Landscape and Forestry and Xiaoman Xie at Shandong Forest Genetic Resources Center for their help in sample collection.

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